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Antigenicity of Proteinases from *Streptomyces griseus* (Pronase)

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The five pronase fractions, A_1, A_2, B, C (trypsin-like), and D (elastolytic), obtained by ion-exchange chromatography, were found to be antigenically distinct. Antibodies to pronase inhibited the enzymic activity of each of the enzyme fractions. Pronase trypsin and bovine trypsin, although resembling each other in enzymic activity and in amino acid sequence around their active sites, did not cross-react antigenically with, nor was their enzymic activity inhibited by, the respective homologous antibodies. Inactivation of pronase trypsin by complexing with soya-bean inhibitor AA, was not associated with a decrease in capacity to precipitate with its antibody. It is assumed that the antigenic sites are located far enough from the catalytic site of the enzyme to allow it to precipitate immunologically even when the catalytic site was blocked.

Commercial pronase prepared from *Streptomyces griseus* contains several proteinases and peptidases (Nomoto, Narahashi & Murakami, 1960*a*,*b*). Purification of pronase by ion-exchange chromatography gave five major immunogenic fractions (Avtalion, Trop, Malik & Pinsky, 1970), one of which (A_1) was not proteolytic. The four other fractions (A_2, B, C and D) were proteolytically active. Fraction B contained an endopeptidase, an aminopeptidase and carboxypeptidases (Trop & Birk, 1970). Fraction C was reported by Trop & Birk (1969, 1970) to manifest a trypsin-like enzymic activity. This enzyme was consequently suggested to possess an active site and some fragment sequences similar to those of bovine trypsin (Wählby & Engström, 1968; Trop & Birk, 1969; Jurasek, Fackre & Smillie, 1969). Another fraction (D) was shown to have elastolytic activity (Trop & Birk, 1970) and to hydrolyse the ester bond of CH_3CO-Ala-Ala-Ala-O-CH_3 (A. Gertler & M. Trop, unpublished work) which was shown to be a specific substrate for pancreatic elastase by Gertler & Hofmann (1970). The work described below includes a further comparison of pronase trypsin with bovine trypsin by using immunologic techniques. In addition, the antigenic purity of the four active proteinases isolated from pronase was investigated.

MATERIALS AND METHODS

Chromatographic separation. Pronase (500mg, B grade, lot 54909, purchased from Calbiochem, Los Angeles, Calif., U.S.A.) was fractionated on a column (5cm × 15cm) of CM-cellulose with a gradient from 10mM-ammonium acetate, pH4.6, to 0.2M-ammonium acetate, pH6.9, as previously described (Trop & Birk, 1968). Further purification of the fractions A_2, B, C and D was achieved by additional separation on CM-cellulose and DEAE-cellulose columns as described earlier (Trop & Birk, 1970). The freeze-dried pronase elastase (D) rechromatographed on CM-cellulose was further purified by applying a solution of 50mg in 50ml of 5mM-potassium carbonate buffer adjusted to pH9.5, to a column (1cm × 50cm) of DEAE-cellulose which had been equilibrated with freshly prepared 5mM-potassium carbonate buffer, pH9.5. The pronase elastase emerged with the first peak eluted with 300ml of the same buffer.

Enzymic activity assays. Proteinase activity was determined by the casein digestion method at pH7.6 as described by Laskowski (1955). Elastolytic activity was assayed by the Congo-Red–elastin method described by Gertler & Hofmann (1967) and modified by Trop & Birk (1970). Hydrolytic activity on benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Serva Entwicklungslabor, Heidelberg, West Germany) was assayed by the method of Erlanger, Kosowski & Cohen (1961) as modified by Trop & Birk (1970).

Enzymic inactivation. For the enzymic inactivation determination, 10μmol of di-isopropyl phosphorofluoridate or L-7-amino-1-chloro-3-toluene-*p*-sulphonamide-2-heptanone (tosyl-L-lysine *p*-chloromethyl ketone) were incubated with 12μg of enzyme at 25°C in 0.5ml of 0.1M-sodium phosphate buffer, pH7.6. The inactivation was tested at 10min intervals by measuring residual benzoyl-arginine-*p*-nitroanilide-hydrolysing activity. Inactivated enzymes for precipitin tests were prepared by 1h incubation at 22°C of 15mg of enzyme in 1ml of saturated dimethylsulphonyl fluoride in 0.9% NaCl, or in 1ml of 0.9% NaCl containing 5mg of soya-bean inhibitor AA; the latter is approximately equimolar to the concentration of the enzymes. Under these conditions, both enzymes are completely inactivated by dimethylsulphonyl fluoride,

whereas bovine trypsin is completely inhibited and pronase trypsin is 85% inactivated by soya-bean inhibitor AA. At greater dilutions of these enzyme–inhibitor systems, the rate of inactivation is markedly decreased.

Immunization procedure. Specific antibodies were obtained by hyperimmunization of rabbits with pronase or bovine trypsin. Rabbits (3 months old) were immunized for 5 weeks by weekly injection into the leg muscles of 1 ml quantities of an emulsion of Freund's complete adjuvant containing 2 mg of pronase or bovine trypsin. At 10 days after the last injection, they were bled and their sera were tested for the presence of anti-pronase or anti-bovine trypsin antibodies by immunodiffusion. Sera were pooled and divided into 1 ml fractions and stored at −20°C.

Isolation of γ-globulin. The γ-globulin fraction of immune and normal rabbit serum was prepared as described by Stelos (1967). The γ-globulin solutions were brought to the original serum volume and stored at −20°C.

Immunodiffusion. Double diffusion was carried out with 1% (w/v) agarose gel in 0.9% NaCl layered thinly on glass slides (7 cm × 7 cm).

Quantitative precipitation. Increasing amounts of antigen were added to test tubes containing 0.5 or 0.3 ml of the pooled antiserum, and the volume was brought to 1.0 ml with 0.9% NaCl, followed by 30 min incubation at 37°C and overnight storage at 4°C. The resultant precipitates were centrifuged, washed twice with cold 0.9% NaCl and dissolved in 3 ml of 0.1 M-NaOH. The extinction of these solutions was measured at 280 nm.

Inhibitory effect of antibodies on enzymic activity. Each enzyme (15 μg) was preincubated at 37°C during 20 min with its corresponding antibody in excess (0.5 ml of 1:5 anti-pronase or anti-trypsin serum γ-globulin fraction), and then tested for digestion of casein or hydrolysis of benzoylarginine nitroanilide. In each case 0.5 ml of 1:5 normal rabbit serum γ-globulin served as control.

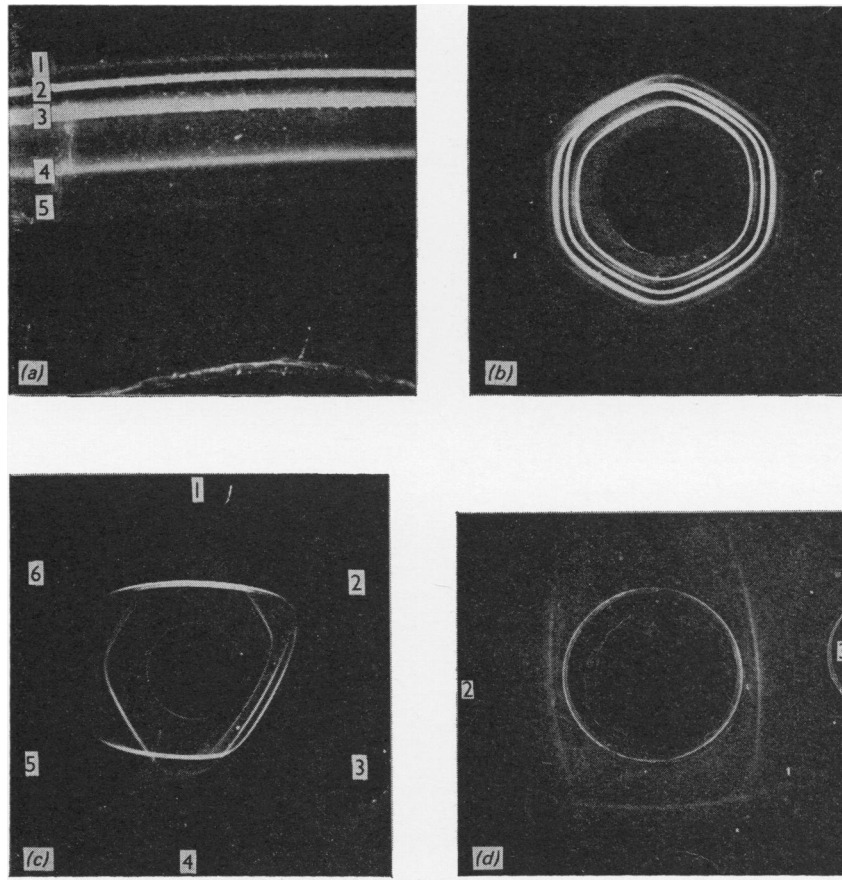

Fig. 1. Immunodiffusion patterns developed in agarose gel: (*a*) bottom well (just visible), pronase antiserum; upper well (off picture), pronase, magnification × 8.7. (*b*) centre well, pronase antiserum; side wells, various lots of pronase, magnification × 1.7. (*c*) centre well, pronase antiserum; (1) pronase fractions A_2 and B; (2) pronase elastase; (3) crude pronase; (4) pronase fraction A_2; (5) pronase trypsin; (6) pronase trypsin and pronase elastase, magnification × 1.1. (*d*) centre well, pronase antiserum and bovine trypsin antiserum; (1) pronase trypsin; (2) and (3) bovine trypsin, magnification × 1.8.

RESULTS AND DISCUSSION

At least five precipitin lines, which probably indicate the existence of five distinct antigenic fractions, were obtained by immunodiffusion of pronase against anti-pronase serum (Fig. 1*a*). This pattern with slight variations was exhibited by most samples of pronase tested (Fig. 1*b*). Each precipitin line was identified respectively with the corresponding line obtained from each of the five chromatographically purified fractions (Trop & Birk, 1969). Major precipitin lines 1, 2, 3, 4 and 5 were therefore respectively recognized as the Trop & Birk (1969) fractions A_1, A_2, B, D and C (Fig. 1*c*).

Pronase trypsin (fraction C) was found to be completely inhibited by di-isopropyl phosphorofluoridate after 10min and by tosyl-lysine chloromethyl ketone after 70min at 25°C (Fig. 2).

The peptide derivative Asp-Ser(DIP)-Gly was previously isolated from the di-isopropyl phosphorofluoridate-inactivated 'pronase trypsin-DIP' (Wählby & Engström, 1968), the serine residue of which was shown to be in the active site of the enzyme. On the other hand tosyl-lysine chloromethyl ketone is known to attack exclusively a catalytic histidine residue in the active site (Mares-Guia & Shaw, 1963). This result indicated the essential role of histidine in the active site of pronase trypsin in addition to the serine group previously implicated. Likewise both amino acids are associated with the well known bovine trypsin active site (Dixon, Kauffman & Neurath, 1958; Walsh & Neurath, 1964; Tomášek, Severin & Šorm, 1965).

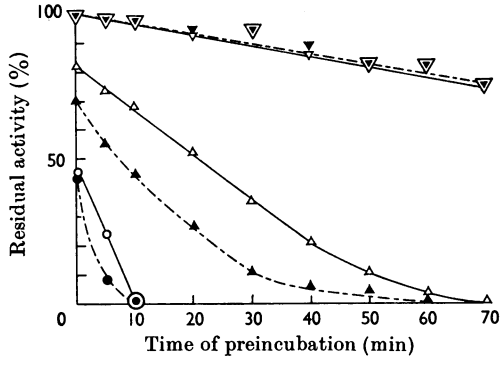

Fig. 2. Residual enzyme activity of bovine trypsin and pronase trypsin on benzoylarginine nitroanilide after preincubation with di-isopropyl phosphorofluoridate (●) and (○) respectively; with tosyl-L-lysine *p*-chloromethyl ketone, (▲) and (△) respectively; and without inhibitor, (▼) and (▽) respectively. The reaction mixture contained 12μg of enzyme and 10μmol of reagent at 25°C.

The precipitin curves of pronase trypsin and bovine trypsin with their respective pooled antisera showed that the maximum amount of precipitate was obtained by reaction of 0.5ml of specific antisera with 750μg of pronase trypsin or 150μg of bovine trypsin. When both enzymes were inactivated by soya-bean inhibitor AA and then reacted with the above equivalent quantities of their respective antisera the amounts of precipitate as compared with the untreated enzymes were somewhat larger (Table 1), probably due to the added weight of soya-bean inhibitor AA in the complex.

Bovine trypsin, both untreated and inactivated with dimethylsulphonyl fluoride, did not cross-react with pronase antiserum. Neither did flocculation appear in the precipitin test of this enzyme against pronase antiserum, nor was any visible precipitin band formed by this system in immunodiffusion. Similarly pronase trypsin showed no immunological cross-reaction with the bovine trypsin antibody. In an immunodiffusion study of bovine trypsin and pronase trypsin against their antisera placed together in the centre well, the corresponding precipitin bands formed did not show a reaction of identity (Fig. 1*d*).

All pronase fractions were completely inactivated in the presence of anti-pronase γ-globulin. No enzymic activity was detected in the reaction mixture containing any of the pronase fractions and pronase antiserum γ-globulin. On the other hand this antibody did not cause any greater inhibition of bovine trypsin than that shown by the serum blank (12% inhibition). No more than 12%

Table 1. *Extinction of redissolved precipitate obtained from pronase trypsin and bovine trypsin and their soya-bean inhibitor AA derivatives, with their respective antisera*

To 0.5ml of antiserum was added 0.5ml of antigen or its soya-bean inhibitor AA derivative in 0.9% NaCl. The quantitative precipitin test was performed as described in the Materials and Methods section.

Antigen	Antisera	Soya-bean inhibitor AA (μg)	E_{280}
Bovine trypsin (150μg)	Anti-(bovine trypsin)	0	0.54
Bovine trypsin (150μg)	Anti-(bovine trypsin)	50	0.60
Pronase trypsin (750μg)	Anti-pronase	0	1.74
Pronase trypsin (750μg)	Anti-pronase	250	1.92

inhibition was obtained when bovine trypsin antiserum γ-globulin reacted with pronase trypsin.

It has already been shown that pronase trypsin resembles bovine trypsin in enzymic specificity, and in the structure of the active site (Wählby & Engström, 1968; Trop & Birk, 1970), especially in the sequences around His-46,* Ser-183 and Asp-177 involved in the active site (Jurasek *et al.* 1969). Similar inhibition of both enzymes by tosyl-lysine chloromethyl ketone and di-isopropyl phosphorofluoridate, described above, showed the catalytic role of histidine and serine in their active centres. In spite of the structural similarity of the enzymes, there was no antigenic similarity as tested by immunologic methods. These phenomena may be explained by the possibility that the antigenic determinants in pronase trypsin are not located at its active site, although the anti-pronase is able to inhibit its enzymic activity. It was already pointed out that inhibition of bovine trypsin and pronase trypsin activity by the soya-bean inhibitor AA involved attachment of the inhibitor molecule irreversibly to the catalytic site of both enzymes (Birk, 1968; Trop & Birk, 1969; Trop, 1969). Therefore, if the region of the antigenic determinant in the above enzymes overlaps the catalytic site, or is adjacent to it, one would expect that the enzyme–antibody interaction in the presence of soya-bean inhibitor AA would be lessened and result in a decrease of precipitation. But, in fact, this did not occur. This supports the above supposition, that the antigenic determinant sites of pronase trypsin are quite different from its enzymic active site.

A similar possible explanation was proposed by Arnon & Schechter (1966) for the observation that natural inhibitors protect completely the catalytic site of bovine trypsin against irreversible inhibition, by plasma inhibitor, but they do not interfere with the precipitation of the enzyme with its antibody. As for the observed inhibition of enzymic activity by interaction with its antibody, this may result from steric hindrance. Further investigation is necessary to clarify this point.

As mentioned by Trop & Birk (1970) the active fractions obtained by chromatography on CM-cellulose and DEAE-cellulose differ in their proteolytic specificity. In the present work marked antigenic differences between these fractions were demonstrated. This finding confirms the concept that pronase is a heterogeneous preparation consisting of independent proteinase molecules.

* The numbers refer to the amino acid sequence of trypsinogen.

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